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PRINCIPAL INVESTIGATOR: Susan Farabaugh

CONTRACTING ORGANIZATION: University of Pittsburgh
Pittsburgh, PA 15213

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Farabaugh, Susan M

E-Mail: Farabaughs3@upmc.edu

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University of Pittsburgh
3520 Fifth Ave
Pittsburgh PA 15213-3320

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14. ABSTRACT

Basal-like/triple negative breast cancers (TNBCs) are characterized by distinctive features most closely resembling basal/myoepithelial cells; however, it is still unclear from which mammary lineage these tumors are derived. Insulin-like growth factor receptor I (IGF1R) is overexpressed and amplified in TNBCs, correlating with poor prognosis, and TNBCs are especially sensitive to IGF1R/Insulin receptor inhibition. Our novel mouse model for retroviral introduction of IGF1R into the mammary gland demonstrates that IGF1R induces basal-like mammary tumors with mixed histologies, including a dramatic expansion of a rare population positive for both luminal (CK8) and myoepithelial (CK14) markers. My preliminary data previously indicated that IGF1R promotes self-renewal and differentiated progeny. The approach proposed in this fellowship is to utilize IGF1R-induced tumor heterogeneity as a model system to help identify how cell of origin and molecular alterations change mammary cell fate to produce heterogeneous mammary tumors. Here, I describe my progress in determining the mechanism of IGF1R-promoted tumor initiation characteristics, whether IGF1R alters self-renewal, and if IGF1R expression in breast tumors correlates with tumor initiation and cell fate markers.

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1. **INTRODUCTION:**

Basal-like/triple negative breast cancers (TNBCs) are characterized by distinctive morphologic, genetic, and clinical features most closely resembling basal/myoepithelial cells; however, it is still unclear from which mammary lineage these tumors are derived. Insulin-like growth factor receptor I (IGF1R) is overexpressed and amplified in TNBCs, correlating with poor prognosis. Consistent with this, we previously reported that TNBCs are especially sensitive to an IGF1R/Insulin receptor inhibitor. Interestingly, our novel mouse model for retroviral introduction of IGF1R to the mammary gland demonstrates that IGF1R induces basal-like mammary tumors with mixed histologies, including a dramatic expansion of a rare population positive for both luminal (CK8) and myoepithelial (CK14) markers. My preliminary data previously indicated that IGF1R promotes self-renewal and differentiated progeny. Together, these data suggest a role for IGF1R in promoting changes in mammary lineage differentiation and fate. The approach proposed in this fellowship is to utilize IGF1R-induced tumor heterogeneity as a model system to identify how cell of origin and molecular alterations change mammary cell fate to produce heterogeneous mammary tumors. Here, I describe my progress in determining the mechanism by which IGF1R promotes tumor initiation characteristics, whether IGF1R alters self-renewal and cell fate, and if IGF1R expression in breast cancer correlates with tumor initiation and cell fate markers.

2. **KEYWORDS:**

IGF1R, triple-negative breast cancer, luminal, myoepithelial, cell fate

3. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Aim 1: Determine the mechanism by which IGF1R promotes tumor initiating cells in vitro (months 0-12) (70% complete)
Aim 2: Examine if IGF1R alters mammary cell fate *in vivo* and the effect this has on mammary tumorigenesis (months 13-30) (20% complete)
Aim 3: Examine whether IGF1R expression in human breast cancers correlates with tumor initiating and cell fate markers (months 30-36) (0% complete)

What was accomplished under these goals?

Aim 1: Determine the mechanism by which IGF1R promotes tumor initiating cells in vitro (months 0-12)

1a: Validate the role of Snail and NF-κB signaling in IGF1R-induced tumor initiating properties

Task 1: Test whether IGF1R induces self-renewal and cell surface marker populations
a. Inhibit NF-kB activity Snail function with inhibitors and shRNA
b. Enumerate mammosphere formation over several generations (passages)
c. Examine the $CD44^+CD24^-$ populations by FACS

My preliminary data suggested that IGF1R expression increases mammosphere formation and expands the CD44+CD24- population of MCF10A cells through Snail and NF-κB signaling. To validate the promotion of tumor initiating properties by IGF1R and the necessity of the EMT factors, we further examined the percentage of $CD44^+$ $CD24^-$ cells and enumerated mammosphere propagation in MCF10A-IGF1R cells over several generations. We re-analyzed the CD44+CD24- population in MCF10A cells expressing the constitutively

active form of IGF1R (CD8-IGF1R) and confirmed IGF1R-induced expansion of this population (Figure 1a). To ensure constitutive IGF1R activation was still functioning in a typical manner, we tested MCF10A cells expressing a wild type form of IGF1R (WT-IGF1R). WT-IGF1R expands this population, but not to the same extent as the constitutively active version (Figure1b). It is important to note that the wildtype-IGF1R cells were not stimulated with IGF-1; and thus, the changes observed are a result of increased basal activation. If we were to stimulate the pathway, we would expect the extent of CD44+CD24- expansion to be closer to that of the constitutively active IGF1R. We observe a similar of CD44+CD24- expansion with constitutive activation of the insulin receptor (CD8-IR) (Figure 1b), suggesting pathway crosstalk which is expected from these two closely related receptors and pathways. We confirmed tumor initiating properties by measuring self-renewal using the mammosphere assay: sphere formation is monitored by growing single cells in low attachment condition in the presence of growth factors but the absence serum (promoting self-renewal and limiting differentiation). IGF1R-induced expansion of the tumor initiating pathway was also demonstrated by increased sphere formation in both regular assay conditions as well as in the absence of the growth supplement B27 (Figures 1c and 1d).

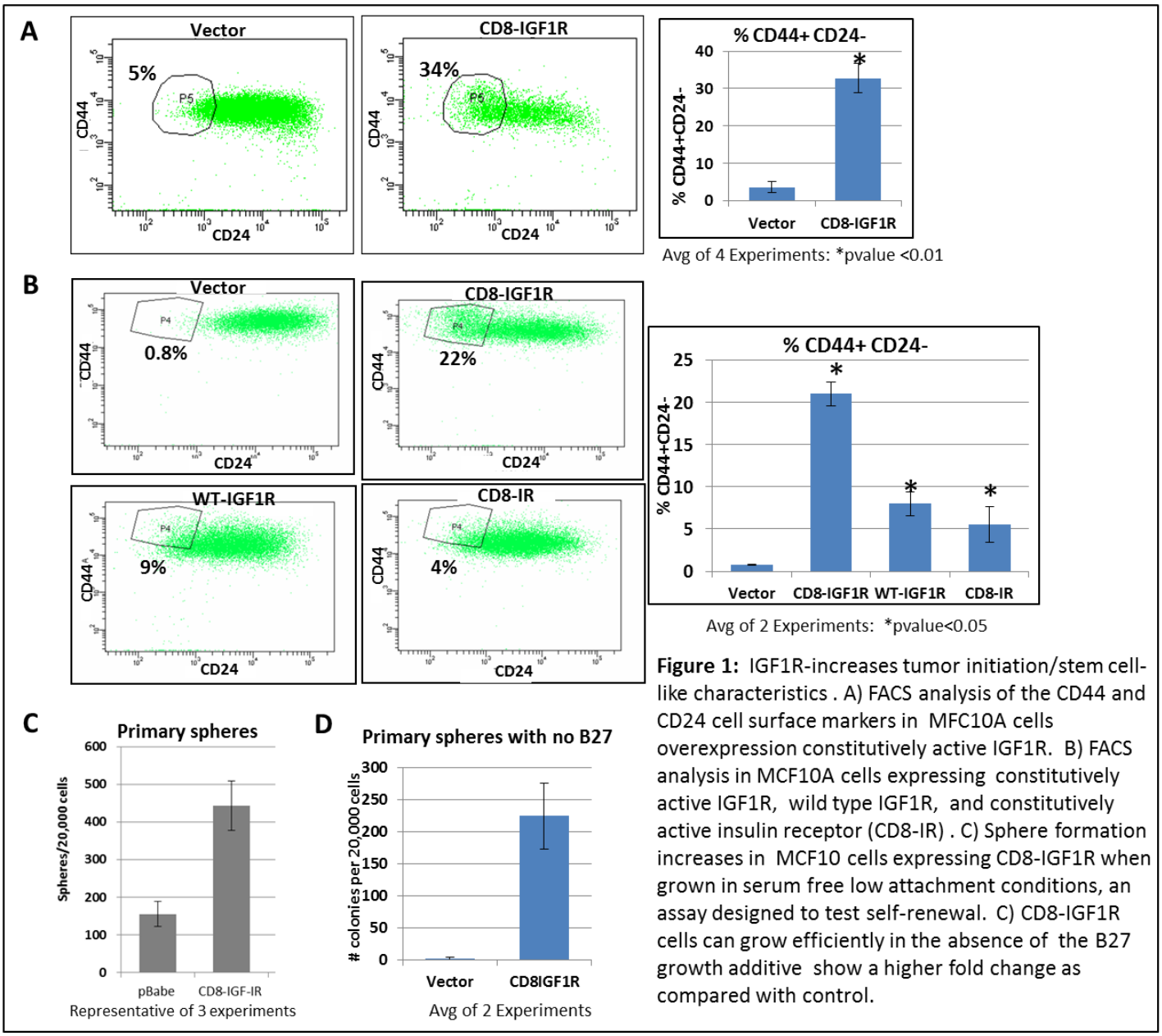

Figure 1: IGF1R-increases tumor initiation/stem cell-like characteristics . A) FACS analysis of the CD44 and CD24 cell surface markers in MFC10A cells overexpression constitutively active IGF1R. B) FACS analysis in MCF10A cells expressing constitutively active IGF1R, wild type IGF1R, and constitutively active insulin receptor (CD8-IR) . C) Sphere formation increases in MCF10 cells expressing CD8-IGF1R when grown in serum free low attachment conditions, an assay designed to test self-renewal. C) CD8-IGF1R cells can grow efficiently in the absence of the B27 growth additive show a higher fold change as compared with control.

Through experimentation of both the CD44+CD24- population and mammosphere formation, we have demonstrated the necessity of both NF-kB and Snail for IGF1R-induced tumor initiating characteristics. The NF-kB pathway was inhibited with the use of an IKK II inhibitor. Expression of Snail was inhibited through expression of a dominant negative Snail stably expressed in the MCF10A-CD8IGF1R cells as well as through Snail siRNA to knockdown the gene. Inhibition of the NF-kB pathway as well as inhibition or knockdown of Snail reversed IGF1R-induced expansion of the CD44+CD24- population (Figures 2a, 2b, and 2d) and primary sphere formation (Figure 2b and 3b). During our experimentation, we observed an IGF1R-induced increase in Slug, also known as Snail2 (Figure3c). FACS analysis of CD8-IGF1R and control cells transfected with either Snail or Slug siRNA demonstrates that IGF1R expands the CD44+CD24- tumor initiating population through upregulation of Snail, but not Slug (Figure 3d). All together, these results demonstrate the IGF1R expands the tumor initiating population through NF-kB and Snail signaling.

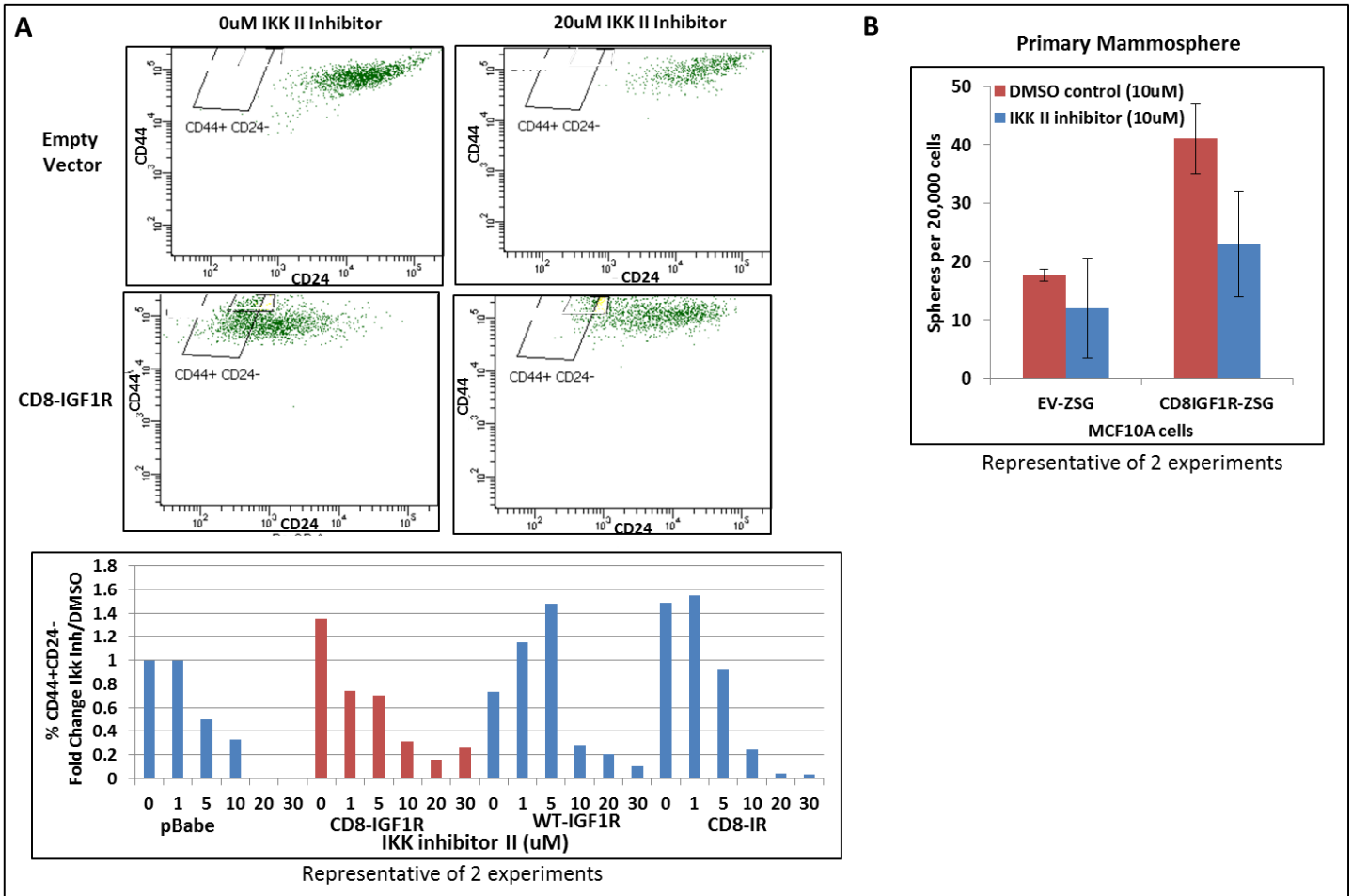

Figure 2: NF-kB is required for tumor initiating properties induced by IGF1R. A) Representative FACS analysis of the CD44 and CD24 cell surface markers with inhibition of the NF-kB pathway with IKK II inhibitors . Quantitative analysis of all inhibitor concentrations is graphed. B) Sphere formation in MCF10-CD8-IGF1R cells requires NF-kB activity when grown in serum free low attachment conditions, an assay designed to test self-renewal.

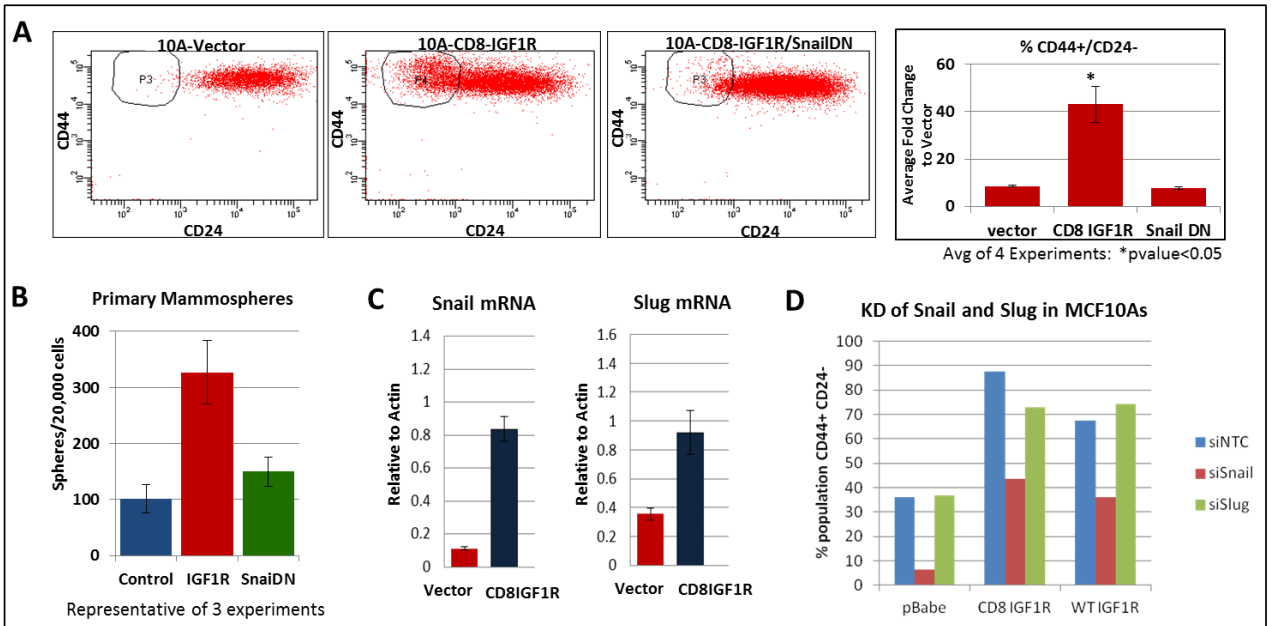

Figure 3: IGF1R expands the CD44+/CD24- population through regulation of Snail and not Slug. A) FACS analysis of the CD44 and CD24 cell surface markers in MCF10A control, MCF10A-CD8-IGF1R, and MCD10A-CD8IGF1R-SnailDN cell lines shows an enrichement in the stem cell – associated CD44+ CD24- population of IGF1R overexpressing cells that is dependent on Snail. B) Sphere formation in MCF10-CD8-IGF1R cells requires Snail when grown in serum free low attachment conditions, an assay designed to test self-renewal. C) In addition to Snail, Slug (Snail2) is overexpressed with expression of activated IGF1R as measured by qPCR. D) FACS analysis with Snail siRNA knockdown reverses the expansion of the CD44+ CD24- population while Slug knockdown does not.

Goals yet to be accomplished for Subaim1a:

We have struggled enumerating the mammospheres over several passages as well as consistent knockdown of Snail and NF-kB in our analyses. We believe the cells have low levels of mycoplasma that may be enhanced in these experimental procedures. To circumvent this, we have remade the IGF1R overexpression in new MCF10A parental cells using the pHIV-ZSG virus developed as a part of Aim2 (see below). These cells have been preliminarily tested and appear to be functioning similarly: IGF1R expands the population of cells with stem-like characteristics in a Snail and NF-kB manner (Figure 4). We will use these cells to complete the mammosphere enumeration and knockdowns. Additionally, we are purchasing viral siRNA vectors (already allotted for in budget) which will circumvent the low transfection rates of MCF10A cells.

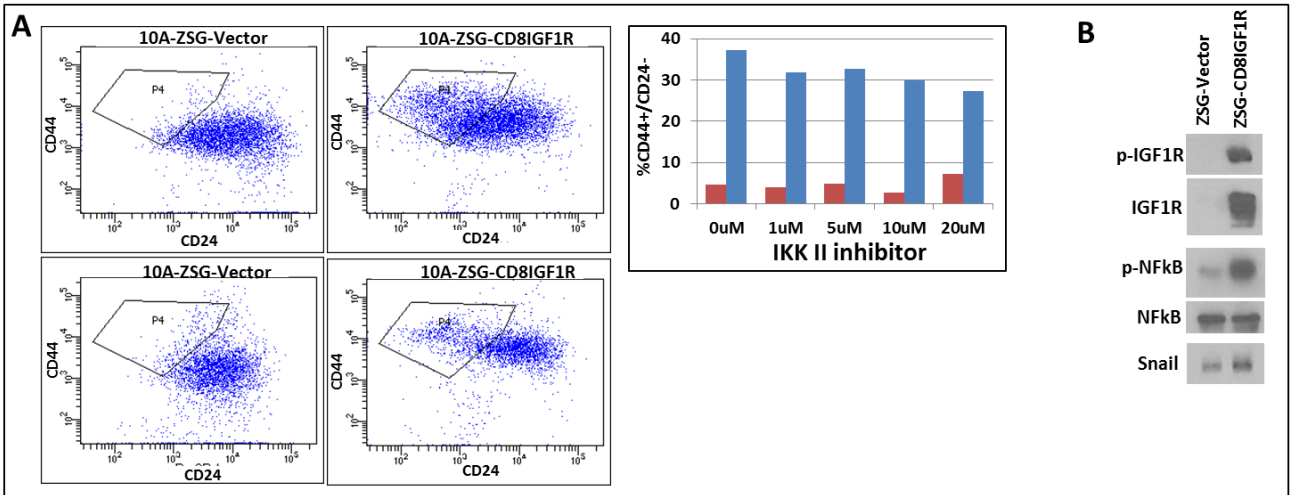

Figure 4: New CD8-IGF1R overexpressing MCF10A cells made using the pHIV-ZSG vector have the same phenotypes as the old pbabe-based cells. A) FACS analysis of the CD44 and CD24 cell surface markers in MCF10A cells infected with pHIV-ZSG-CD8-IGF1R and pHIV-ZSG empty vector. Cells were infected, sorted for ZSGreen, treated with IKK II inhibitor at increasing concentrations, stained, and analyzed for CD44+CD24-. B) Western of MCF10A-ZSG-IGF1R showing total and phospho IGF1R expression as well as activation of NF-kB and increased expression of Snail.

Aim 1a: Task 2: Test the in-vivo tumor initiating ability in the absence of Snail and NF-kB activity

a. Inhibit NF-kB activity and Snail with p65 and Snail shRNA (n=125)

b. Serial dilution injection into NOD SCID mammary glands with weekly palpation

This task is to test the in-vivo tumor initiating ability in the absence of Snail and NF-kB activity. MCF10A cells are not easily transformed. MCF10A-CD8IGF1R cells do form tumors in immunocompromised mice, which is why this experiment was originally proposed, but the tumors are very small and soft, unlike typical mouse mammary tumor formation. They do not grow robustly. We can run a pilot experiment to test the reversal of this growth upon knockdown of Snail and NF-kB, but we do not believe the tumors are robust enough to test a substantial difference in growth. To circumvent these issues, we are in the process of finding another cell line model to test in vivo requirement for Snail and NF-kB. We are currently looking for a breast cancer cell line model which depends on the endogenous activity of IGF1R for tumorigenesis to replace the MCF10A overexpression model. We have used sequencing data to choose 3 breast cancer cell lines that contain higher IGF1R expression and have tested these cells by western blotting for expression and activation of IGF1R (Figure 5). Thus far, we believe the BT549 cell line may be a good replacement option. Although all cell lines express higher endogenous levels of IGF1R, the BT549 cells have high levels of phosphorylated IGF1R, suggesting high pathway activation. We have preliminarily begun to test the BT549 cell line to determine if the activation of IGF1R is lending to increased NF-kB activity. While we do not see a difference in active phosphorylated NF-kB between the tested cell lines, we discovered that NF-kB activity of BT549s is highly dependent on IGF1R expression (Figure 5). In contrast, NF-kB activity was not altered by lack of IGF1R in the other tested cell lines (data not shown). The lack of activated IGF1R and lack of IGF1R-dependent NF-kB activity suggests these other cancer cells lines are driven in non-IGF1R manner. In conclusion, our results suggest that IGF1R may be the driving tumorigenic force in BT549s and would be a great model to replace the MCF10As in testing tumor initiation.

While discovering the BT549 cell line, we also started working on a second model. This option is to take a cell line that is slowly tumorigenic in mice, such as MCF7s, and overexpress IGF1R. Based on our results from MCF10A-IGF1R cells, we expect the mouse tumors to progress more quickly with constitutive activity of IGF1R and to be dependent on Snail and NF-kB in a similar manner as observed previously. This system would be very similar to the MCF10A system we are currently using and we can then utilize this cell line in the exact same manner as proposed for MCF10A system. If the BT549 model fails, for example does not form tumors in mice, we will continue to pursue the MCF7 model.

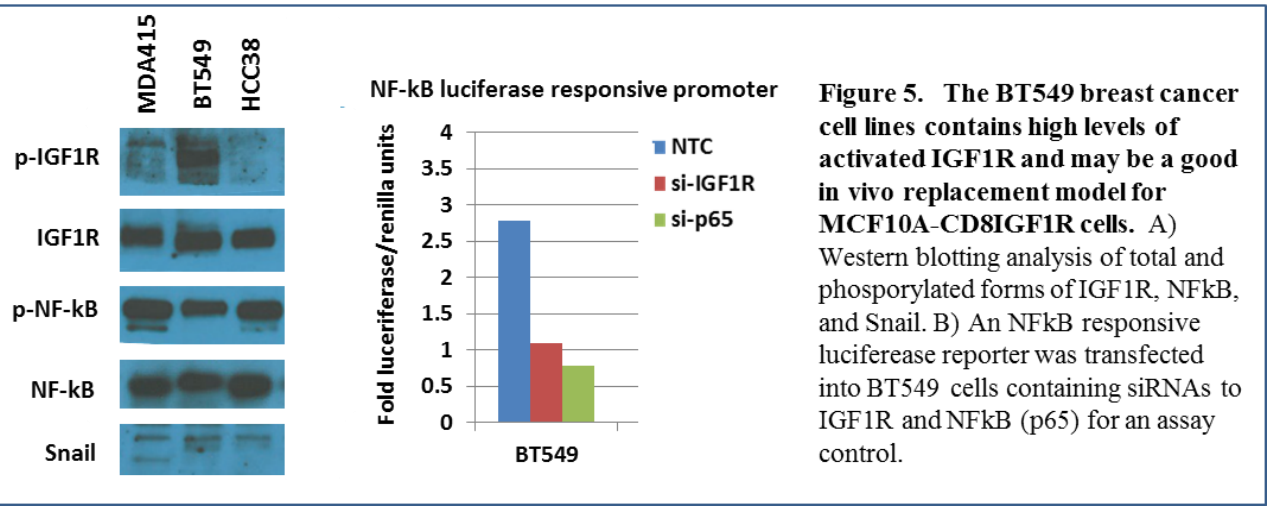

Figure 5. The BT549 breast cancer cell lines contains high levels of activated IGF1R and may be a good in vivo replacement model for MCF10A-CD8IGF1R cells. A) Western blotting analysis of total and phosporylated forms of IGF1R, NFkB, and Snail. B) An NFkB responsive luciferease reporter was transfected into BT549 cells containing siRNAs to IGF1R and NFkB (p65) for an assay control.

Aim 1b: Extend the role of Snail and NF-κB signaling in IGF1R-induced tumor initiating properties using primary human and mouse MECs (months 6-12)

Task 1: Test whether IGF1R induces self-renewal and cell surface marker populations

a. Obtain HMECS (Invitrogen) and harvest/isolate primary mouse MECS (n=20)

b. Infect with GFP-IGF1R or control lentivirus and sort for GFP expression

c. Measure production of tumor initiating characteristics as outlined in Subaim1a

We have successfully cloned CD8-IGF1R into the pHIV-ZSG lentiviral vector, collected highly concentrated virus and tittered. Tittering was performed by infecting 293T cells with viral dilutions, performing FACS, and calculating the infectious units (Figure 6). This highly concentrated tittered virus was produced and is necessary for Aim2 but has been useful for transducing cells in this aim.

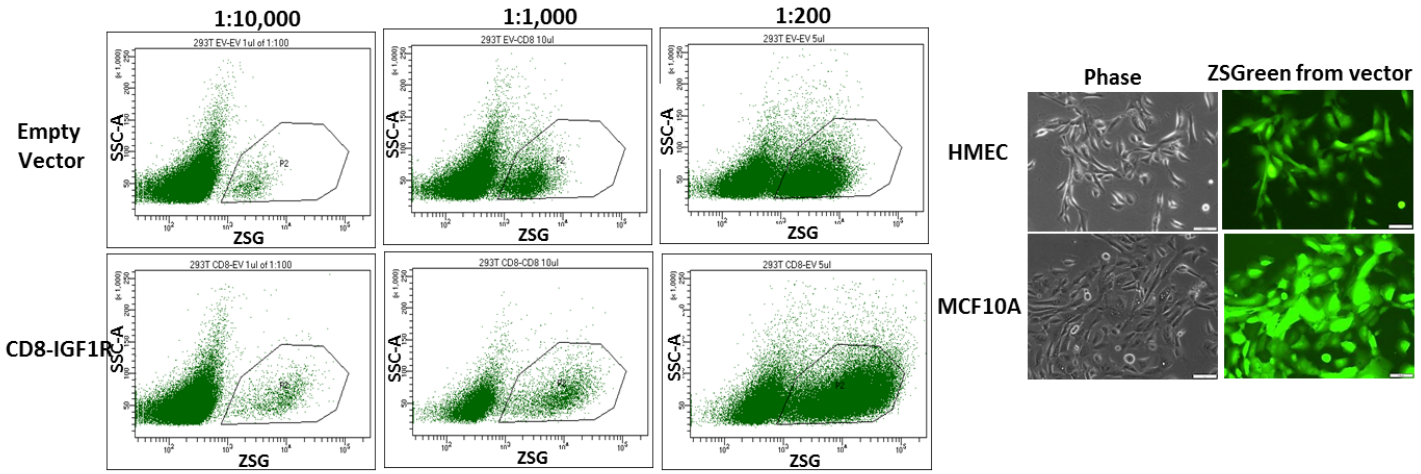

Figure 6: Successful production, concentration, titering, and infection of pHIV-ZSG virus. A) FACS analysis of pHIV-ZSG viral, both empty vector control and CD8-IGF1R, titered into 293T cells. B) Phase and fluorescent imaging of live HMEC and MCF10A cells infected with pHIV-ZSG empty vector at an multiplicity of infection of 10. The MCF10A cells pictured here are post-sort for ZSGreen expression while the HMECs were not sorted.

Aim1b: We infected HMEC cells with either empty vector control or CD8-IGF1R containing virus at a multiplicity of infection (MOI) of 1, 5, or 10. We originally only attempted an MOI of 10 (5-20 is suggested for primary cells); however, we observed a dramatic change in morphology only in the CD8-IGF1R transduced cells. The cells went from having a scattered phenotype to stacking up and staying in individual balled colonies (Figure 7a). The cells did not survive well through the first set of attempted experiments. We believe that this large amount of constitutively active IGF1R is unhealthy for these non-cancerous primary cells. These HMEC cells will not be sorted before use as they are more sensitive to handling and need to be made fresh for each experiment. Thus, we need a highly efficient (to avoid sorting) but healthy transduction. A tittered MOI of 5, as based on 293T tittering units, nicely transduces most cells and appears to be a healthier dose for the HMECs. Although western analysis suggests total NF-kB expression may decrease, IGF1R increases the activated phosphorylated form of NF-kB, (Figure 7b). Further experiments are currently ongoing to test self-renewal, tumor initiating capabilities and dependence on NF-kB and Snail.

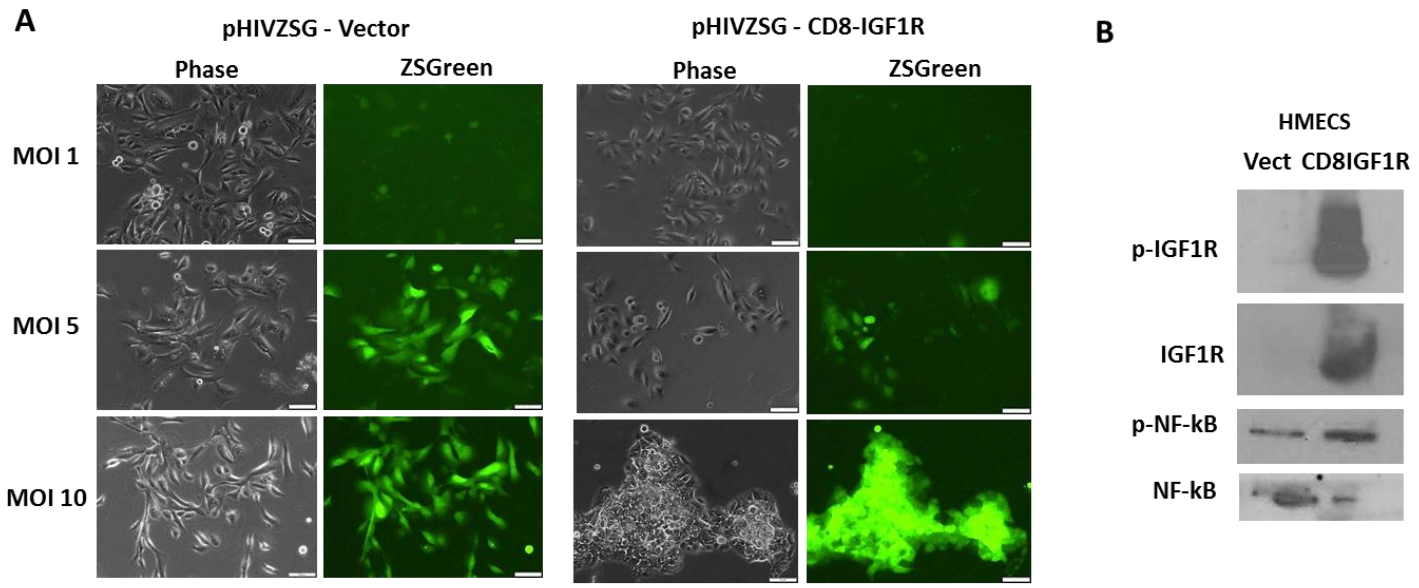

Figure 7: HMECS overexpressing CD8-IGF1R change morphology and increase NF-kB activation. A) HMECs were infected with pHIV-ZSG lentivirus containing either vector control or CD8-IGF1R at different multiplicity of infections (MOIs). Images show phase and ZSGreen expression produced from vector. B) Overexpression of total and phospho-CD8-IGF1R in the HMECS and increase in phospho-NF-kB is demonstrated by western.

We have also began work on the mouse MECs. We have optimized and can now efficiently harvest the mouse mammary glands, dissociate, lineage positive deplete, and plate. Several experiments have been performed with these cells as part of Aim2. For this Aim, the first two attempts at infecting have resulted in large amounts of death so we are currently working on calculating a better titter.

Aim1b: Task 2: Test in vitro and in vivo tumor initiating ability in the absence of Snail and NF-kB activity (3 months)

a. *Obtain/harvest and infect HMECs and mouse MECS with IGF1R as in Task1*
b. *Inhibit NF-kB activity Snail function with inhibitors and shRNA*
c. *Measure production of tumor initiating characteristics as outlined in Subaim1a*
d. *Serial dilution injection into NOD SCID mammary glands with weekly palpation*

As stated above, the HMEC cells are ready and testing has begun. IGF1R expression in these cells does enhance NF-kB activity (Figure 7b), suggesting the HMECS will react similarly to IGF1R expression as the breast cell lines. Experiments are currently ongoing, testing Snail and the necessity for NF-kB. The mouse MECS should be ready shortly.

Goals yet to be accomplished for Subaim 1b:
We have the HMEC system optimized and can efficiently transduce fresh batches of primary cells as needed for experiments. We are currently testing the tumor initiating properties and the necessity for NFkB and Snail (Task 1c and 2b-2d). The isolation of mouse MECS is optimized and tumor initiating experiments will begin shortly (Task 1c and 2b-2d). Serial dilution injection into mice will follow shortly thereafter.

Aim 2: Examine if IGF1R alters mammary cell fate in vivo and the effect this has on mammary tumorigenesis (months 13-30)

2a: Determine if IGF1R promotes stem/tumor initiating properties and creates multi-lineage progeny from differentiated mammary cells (months 13-24)

Interestingly, two mouse models in our lab show that IGF1R forms tumors with mixed histologies and/or an expanded putative bi-progenitor population (Fig1 & Fig2). This data, as well as the expansion of tumor initiating characteristics, suggests that IGF1R may affect progenitor cell fate and/or the differentiation ability of progenitor cells as well as more differentiated cells. In this aim, we wish to determine if aberrant expression of IGF1R is able to 1) promote stem/tumor initiating properties in all mammary lineages and/or 2) create a multi-lineage progeny from a single differentiated lineage. To determine these, we are expressing IGF1R in mouse primary MEC lineages and ask if its expression alters the self-renewal/tumor initiating properties and progeny/differentiation status of each mammary lineage.

Aim2a: Task1: Test whether IGF1R alters tumor initiation properties and differentiation capability of each MEC lineage population in vitro (3 months)

a. Harvest and isolate primary mouse MECs, sorting by lineage (n=80)

b. Infect each lineage with IGF1R or control lentivirus and seed cells into 3 assays:

1. Analyze organoid formation and appearance from single cells in 3D Matrigel

2. Analyze differentiation potential, plating single cells onto collagen coated coverslips and staining for lineage-specific differentiation

We are first using *in vitro* methods to test both the self-renewal and differentiation status of each mammary lineage upon expression of IGF1R. We have optimized our ability to isolate primary mouse MECs from mammary glands of 8-10 week old FVB/N mice. This process includes dissociation of the cells and removal of the lineage positive population (blood cells, lymphocytes, etc). We have optimized the staining and FACS sorting of the mammary populations by lineage. However, for the initial experimentation, we have chosen to work with whole mammary populations to first optimize our experimental techniques. These optimization experiments are all outlined below. As demonstrated in Aim 1b, we have successfully cloned CD8IGF1R into our viral vector, produced large amounts of virus, concentrate, titter, and successfully transduced several cell types (Figure 6). We are currently tittering the mouse MECS with the CD8-IGF1R virus to determine an appropriate MOI. The first experimental attempt utilized too concentrated of a virus. Once MOI is determined, we will move on to the in vitro assays outlined in Task1b.

While optimizing for these more involved experiments, we chose to work with CD8-IGF1R mouse mammary tumors with the idea that these initial assays will thus also give us usable data on the lineages present in IGF1R tumors versus normal mammary glands. These tumors are readily available in the lab from our transgenic colony and 1 tumor from 1 mouse gives us exponentially more cells than can be isolated from harvesting 4 sets of mammary glands from 5 mice. These traits have improved our efficiency at optimizing while limiting our mouse use. As described below, by optimizing with this system, we also were able to obtain a significant amount of data that can be combined with the results we now obtain from analyzing each mammary epithelial lineal population.

Initially, we analyzed lineages induced in the different transgenic tissues through use of FFPE sections. As we expected the tumors to be composed of a much more complex mixture than the normal mammary gland, we used ErbB2/HER2-induced tumors for a luminal-like control and a p53null claudin low tumors for a more basal/progenitor enriched control for mammary lineage comparisons. The ErbB2 tumors were obtained part

way through the optimization so they are not included in all of the experiments and figures. As expected, the ErbB2 tumor primarily expresses the luminal marker cytokeratin 8 (K8) (Figure 8a). The p53 null tumor was composed much differently and expressed only small amounts of the luminal marker. The CD8IGF1R pre-neoplastic gland and CD8-IGF1R tumors (both from transgenic mice) expressed much higher levels of the keratin 8, luminal, marker and keratin 14, myoepithelial marker as compared with the wildtype mammary gland and all other preneoplastic glands and tumor types. Importantly, we observe the same expansion of the putative bi-progenitor population in these tumors as we previously observed in other IGF1R mouse models (Figure 8a, CD8IGF1R Tumor panel). Interestingly, the CD8-IGF1R tumor initially appears to express a higher proportion of luminal cells and primarily only shows co-myoepithelial staining in the luminal cells.

As part of our optimization, we harvested the tumors and glands, dissociated into single cells. To analyze tumor lineages among these dissociated cells, we and immediately cytospun onto a slide, staining for k8 and k14. we observe a large proportion of myoepithelial cells in the CD8-IGF1R tumor (Figure 8d). As expected and for comparison, the normal mammary gland has a proportional amount of luminal and myoepithelial cells while the HER2/ErbB2 tumor shows predominately luminal staining. These results suggest that CD8-IGF1R is promoting a mixed lineage tumor, composed of both luminal and myoepithelial cells.

As mentioned above, we have optimized isolation of mouse MECS. As we will need to sort the mammary glands by lineage populations, we have taken the harvested, dissociated, lineage negative cells and stained them with various cell surface lineage markers. The FACS analysis shown in figure 8b, panel 1 demonstrates our ability to dissociate, stain the cells, and sort them to separate the luminal cells (blue) from the basal/myoepithelial (purple) and progenitor (red) cells. This lineage sorting step is sufficiently optimized for the start of in vitro and in vivo lineage experiments. The markers used in this experiment to denote these populations have been determined in other laboratories. We have successfully used many more cell surface markers to further identify the lineages, but are using the basic CD24 and CD49f markers as an initial first step as the other combinations begin to get quite tedious. When we analyze the pre-neoplastic glands with the CD24 and CD49f lineage markers, the cells do not separate very well and we observe one large population rather than individual populations (Figures 8b and 8c). This large population shifts slightly with every experiment. It has been difficult to determine what this large population represents for lineage delineation. It is possible that the cell surface markers in the tumor have been affected from the environment and have changed. We observe similar results with ErbB2 tumors (not shown).

As these CD8IGF1R tumors are readily available, we also tested tumor initiating properties in these samples to expand and support the data from Aim1. Dissociated CD8-IGF1R tumor cells produce significantly more spheres in low attachment, serum-free conditions than both normal/wild type mouse mammary cells and the p53 null tumor comparison (Figure 9), suggesting CD8-IGF1R tumor cells possess a much higher capability to survive and self-renewal in these conditions. This finding is interesting as the p53null claudin low tumors are typically believed to be a more basal/progenitor and tumor initiating tumor type.

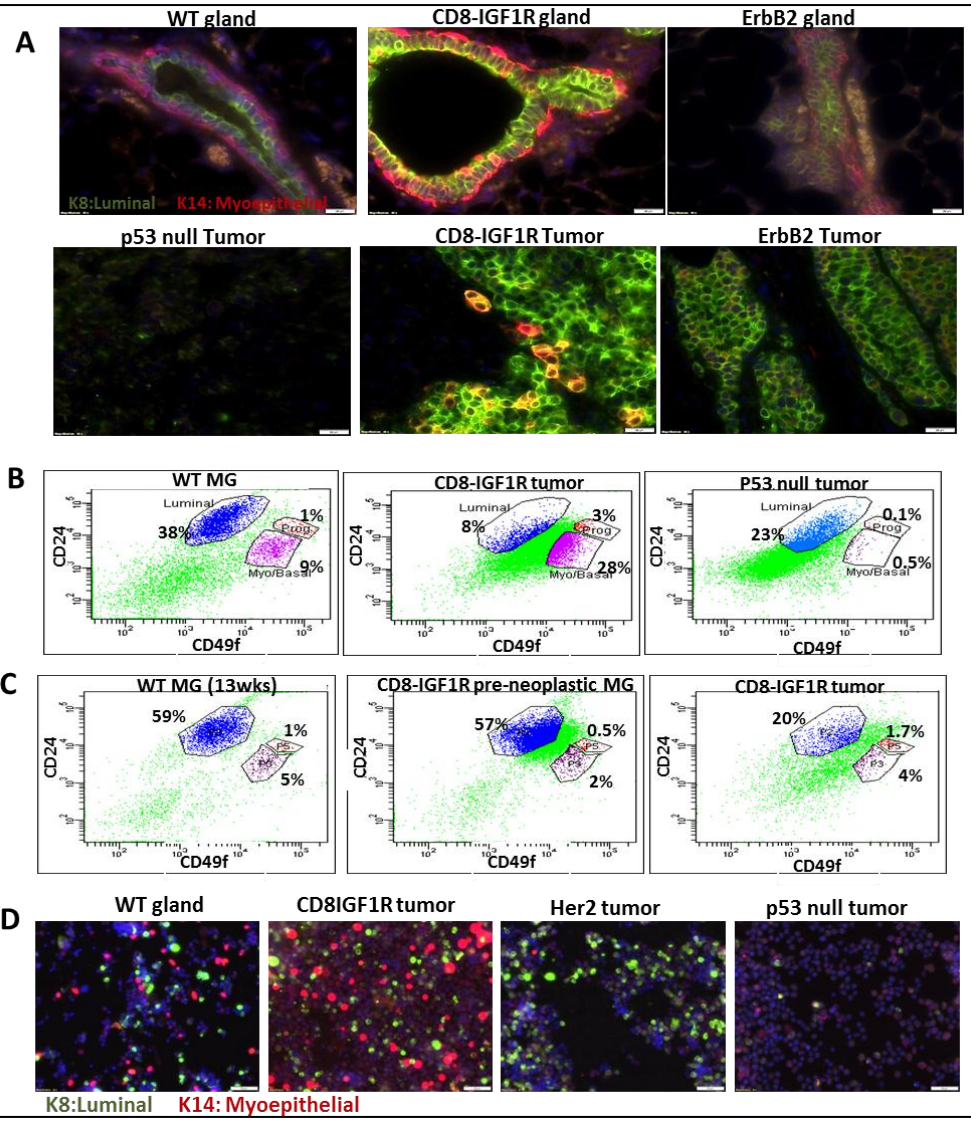

Figure 8: CD8-IGF1R pre-neoplastic glands and tumors have stronger luminal and myoepithelial staining. A) immunofluorescence staining of wild type mammary gland, CD8-IGF1R pre-neoplastic, ErbB2 preneoplastic, and tumors for p53 null, CD8IGF1R and ErbB2 with the luminal marker Keratin 8 (green) and the myoepithelial marker Keratin 14 (red). B) Tumor tissue was dissociated, lineage positive cells removed, and FACS analyzed for CD24 and CD49f to separate the luminal (blue), basal/myoepithelial (purple), and progenitor (red) populations. D) Dissociated tumors were cytospun unto slides and stained with keratin 8 (green) and keratin 14 (red).

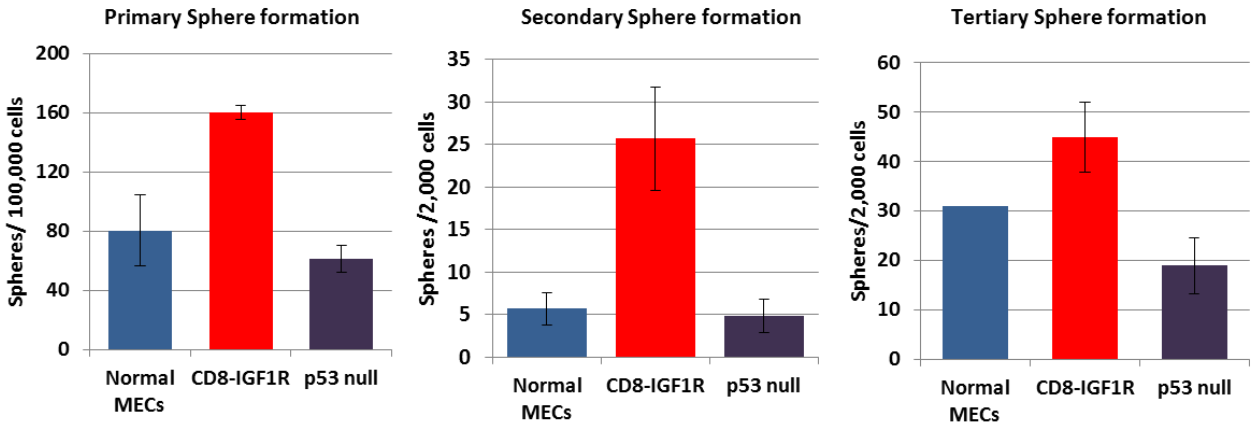

Figure 9: IGF1R tumor tissue has increased tumor initiation/stem cell-like characteristics as compared to Normal MG and p53 null tumors. Tissue was harvested from Normal mouse MG, CD8-IGF1R tumors, and p53 null claudin low tumors. Dissociated tissue/tumor cells were plated into serum free, self-renewing, low attachment conditions and grown for 7-10 days with addition of self-renewal promoting media. Spheres were dissociated a replated for Secondary and then again for Tertiary spheres.

To optimize experimental procedures for Task1 2b assay 2, we used the CD8IGF1R tumor cells to analyze differentiation potential. To test differentiation potential cells were first enriched for stem-like self renewal properties through growth in low attachment serum-free mammosphere assays as performed for Figure 9. To produce a secondary mammosphere assay, the primary spheres are harvested, dissociated, and re-plated as single cells into a new assay. This process can be repeated multiple times for tertiary spheres and upward. For differentiation, stem-like enriched cells are dissociated into single cells and plated into 1 of 2 assays. 1) Plated onto collagen coated coverslips, grown for 5 days, covered with Matrigel, and grown in prolactin to promote differentiation. 2) Plated as single cells into a culture of irradiated fibroblasts with differentiation-promoting media conditions. Both of these assays have been demonstrated to promote lineage-specific differentiation. We optimized both of these assays. CD8-IGF1R tumor cells as well as control wild-type mammary glands were harvested, dissociated, and plated into mammospheres. These enriched cells were enumerated to secondary and tertiary spheres. Either the secondary or tertiary spheres were harvested and plated onto collagen coated coverslips or irradiated fibroblasts as single cells to form single colonies. We observed mammary lineage differentiation in each of these assays (Figure 10). For the irradiated fibroblast co-culture, we stained the cells with crystal violet and designated each colony as appearing luminal, myoepithelial, or mixed lineage. Normal wildtype mammary gland cells isolated from secondary spheres formed a fairly even number of luminal versus myoepithelial colonies. For tertiary stem-like enriched normal cells, many more luminal colonies were formed. In great contrast, CD8-IGF1R tumor cells isolated from both secondary and tertiary spheres differentiated into many more myoepithelial colonies than the other two lineage types. These results suggest that IGF1R may be promoting the differentiation of the myoepithelial lineage. Additionally, this supports the mouse and clinical data suggesting that IGF1R drives more basal-like tumors.

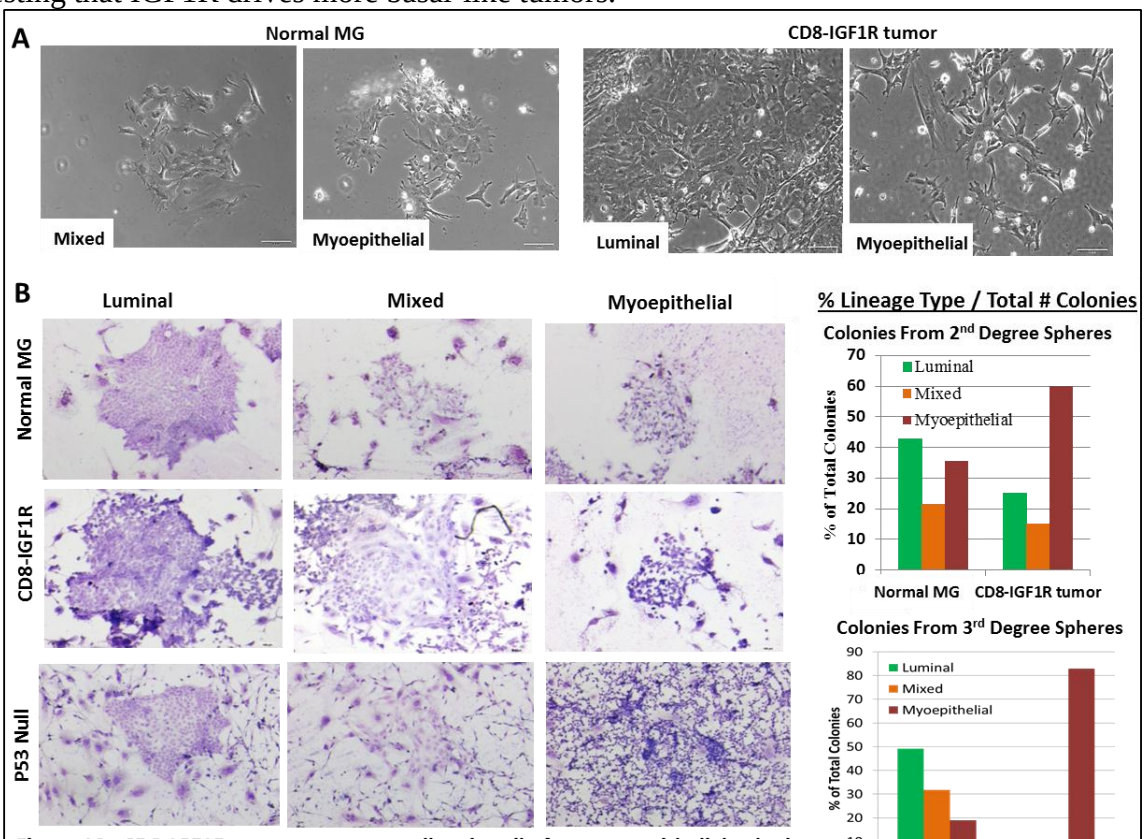

Figure 10: CD8-IGF1R mammary tumor cells primarily form myoepithelial colonies under differentiating conditions. A) CD8-IGF1R transgenic mouse tumors, p53 null tumor grafts, and normal mammary glands from 10wk FVB/N mice were dissociated and plated into primary and then secondary and tertiary mammospheres. Secondary and tertiary mammospheres were dissociated and plated as single cells onto irradiated fibroblasts under differentiating media conditions and grown for 10 days. Differentiated colonies were stained with crystal violet and counted, distinguishing between luminal, myoepithelial and mixed phenotypes by morphological differences. Images are taken at 11.5x.

Aim 2a: Task 2: Test whether IGF1R can differentiate or de-differentiate each MEC lineage population in vivo to repopulate the mouse mammary gland (8 months)

a. *Harvest and isolate primary mammary MECs, sorting by lineage (n=130)*
b. *Infect each with IGF1R/control lentivirus and transplant into cleared fat pad (n=175)*
c. *Harvest mammary glands of 5 mice at 4, 7 and 10 weeks, allowing 10 mice to grow and develop for 7-10 months or until tumors are formed*
d. *Analyze outgrowths through IHC staining for lineage markers and mRNA analysis for lineage specific gene expression (basal factors vs luminal associated)*
e. *Calculate in vivo differentiation/de-differentiation ability by comparing the percentage of subpopulations present in each reconstituted gland*
f. *Analyze a subset of mammary glands/tumors for 3D organoid formation in Matrigel to determine functional capacities of the derived lineages*

It has been demonstrated that just 1 mammary stem cell is able to re-grow all mammary lineages. In task 2, we wish to determine if IGF1R affects the differentiation potential and outgrowth of each mammary epithelial lineage population. This is determined by performing reconstitution experiments where the mouse gland is cleared of all epithelial cells and new cells are added left to regrow. These experiments involve harvesting MECs as discussed above, sorting for each lineage population, and infecting them with CD8-IGF1R lentivirus, all of which was discussed above. The next step will be to transplant the transduced cells into cleared mammary fat pads. We needed to optimize several steps of this reconstitution experiment: 1) our ability to efficiently clear the fat pad of invading epithelial cells, 2) precisely inject only 10ul of concentrated cells, 3)analyze the ability to stain the same gland for both reconstitution and lineage analysis. To test and optimize these steps, we ran a small pilot test with 10 mice. We first harvested and dissociated mouse MECs from wild type mammary glands of 10 week old mice and tumors from both CD8-IGF1R and p53 null tumors as previously described. Pre-pubertal, 21 day old mice underwent surgery to remove the epithelial cells from around the nipple area – if left, these cells would invade into the fat pad and form the normal mammary outgrowths. We then injected 10ul of the dissociated wild type or tumor cells into each fat pad that was just cleared of invading epithelial cells. Cells were left to grow for 4-8 weeks to branch and outgrow the gland. As we were using tumor cells, we needed to harvest these mammary glands before tumors formed, thus the 4-5 week harvest. As depicted in Figure11a, we were able to completely clear the mammary fat pads of all epithelial cells and obtained efficient reconstitutions and mammary outgrowths of normal cells.

The third reason for optimizing with this pilot was to test the ability to perform immunofluorescence after carmine staining. The carmine staining is needed to stain the epithelial cells of the whole mounts so we can determine the ability of the IGF1R expressing cells to re-outgrow and recapitulate a normal mammary gland. We also need to examine each outgrowth on a cellular level, preforming immunofluorescence for co-expression of lineage markers to determine if IGF1R affects the lineage of the outgrown mammary branches. The red carmine stain was a concern for massive autofluorescing during immunofluorescent imaging. If we cannot perform both stainings, we would have to double the number of mice used so half the glands could be analyzed for reconstitution while the other half analyzed for lineage composition. Fortunately, our results demonstrate that all 3 of our immufluorescent channels are usable (Figure 11). The green secondary always autofluoresces at a low level in this processed samples. The far red channel is depicted overlayed on the red channel. While staining was not as bright for this channel, autofluorescence was minimal.

Now that we have optimized each step of this Aim, we have begun to expand our experiments to test our original questions. The mice for the initial reconstitution experiment are already ordered and have arrived. Within the next week, the mouse MECs will be harvested, infected with CD8-IGF1R or control virus, and injected into cleared fat pads. Following the outcome of this experiment, we will then proceed to lineage sorting and repeat.

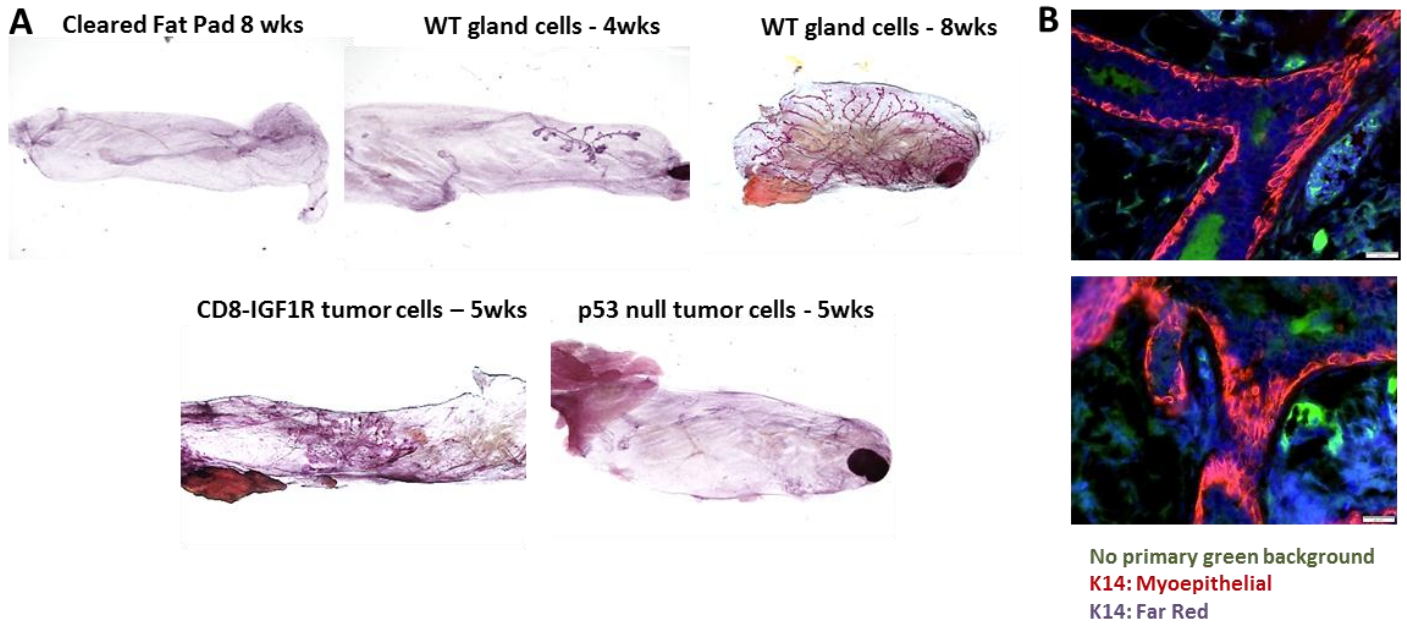

Figure 11: Cleared Mouse Fat Pads can successfully be reconstituted with wild type mammary gland cells and stained for immunofluorescence after use of carmine. A) Mammary glands (10 wk old mice) or tumors (3-5 months) were dissociated, lineage positive cells removed, and 10,000 cells were injected back into a cleared mammary fat pad of 21 day old mice. Glands were left to reconstitute for 21 days, harvested, carmine stained, and imaged. B) The carmine stained WT glands were embedded and the FFPE samples sectioned and stained for Keratin 14 (K14) by immunofluorescence, using red and far red secondaries to determine carmine-induced background interference.

In Task 2d, we propose analyzing our IGF1R-induced lineage outgrowths by determining the expression of various lineage-specific genes. While waiting to obtain these lineage outgrowths, we preliminary tested this panel against our tumor tissues. Mammary gland or tumor tissue was harvested, snap frozen, and mRNA isolated. Real time PCR was used to determine the expression of each gene. CD8-IGF1R tumors expressed much higher levels of luminal progenitor-associated genes than any other normal or tumor tissue, including the luminal-associated HER2/ErbB2 tumors (Figure 12). As expected and in contrast to the luminal association of CD8-IGF1R, the p53 null claudin low tumors, our basal-like control, expressed high levels of basal/myoepithelial-associated genes.

These results counter results obtained in Figure 10. Figure 10 demonstrated differentiation potential of stem-enriched IGF1R tumor cells to favor the myoepithelial lineage in vitro. Here, we suggest the transcription factors driving this tumor may be of luminal origin. It is important to keep in mind that the differentiation experiment utilized stem-like enriched cells while the lineage-associated gene experiment analyzes the tumor mass. This may suggest that the differentiation potential of the stem-like cells is a separate entity from the gene expression of the main tumor mass. Another suggestion is that CD8-IGF1R is acting similarly as human BRCA1-mutant tumors which are basal-like tumors recently discovered to originate from luminal progenitors. Our proposed experiments of analyzing IGF1R in each separate mammary lineage should help us to answer this question.

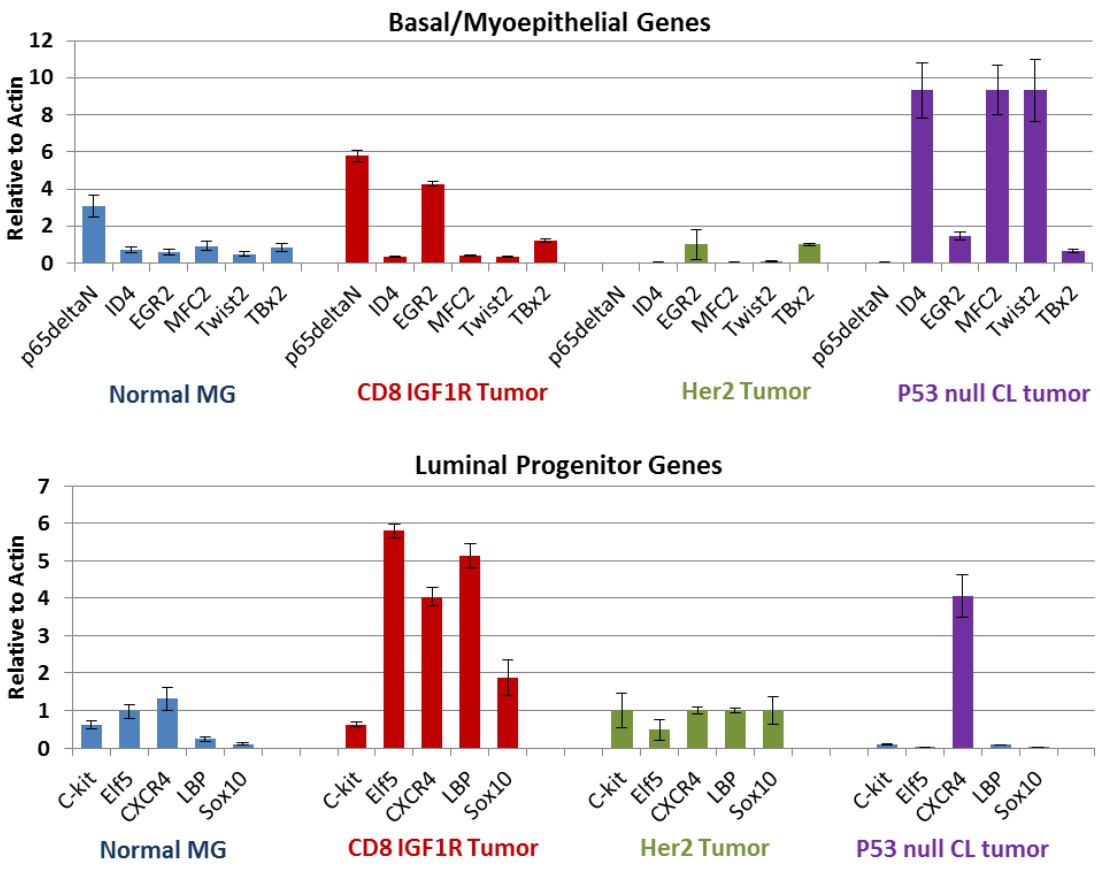

Figure 12: CD8IGF1R tumors express more luminal progenitor genes while p53 null (claudin low) tumors express more basal/myoepithileal –associated transcription factors. Frozen Normal mammary gland s or tumors were crushed and RNA harvested. qPCR was performed using a lineage-associated gene panel.

In conclusion for Subaim 2, both myoepithelial/basal and luminal markers and genes are upregulated in CD8-IGF1R tumors as compared to other mammary tissues. The CD8-IGF1R tumors cells enriched for tumor stem-like properties predominately differentiate into myoepithelial-like colonies. In contrast, CD8-IGF1R tumor cells harvested directly (not enriched for stem properties) express predominately luminal progenitor-associated genes.

In method summary, we have demonstrated the optimization of each step necessary to complete Aim2a. Analyzing the outgrowths of each IGF1R overexpressing lineage will help us determine the control of IGF1R on the fate of each lineage. With this model, we will be able to determine the potential of each lineage over time rather than just analyzing the resulting mass of cells in the tumor.

Aim2a goals:
This Aim is ahead of schedule.
Task 2a is completed and demonstrated above in Task1. Task 2b to infect and transplant into a cleared fat pad to measure the ability to reconstitute the mammary gland is currently underway. There is nothing to report on the rest of these specific tasks.

Subaim 2b: Determine how IGF1R lineage-restricted expression affects tumor initiation and tumor heterogeneity in an intact mouse model that parallels human tumorigenesis (months 21-30)

Task 1: Test tumorigenetic ability of IGF1R in each lineage using transgenic mice with lineage-specific TVA-tagged promoters to target IGF1R expression (9 months)

a. *Expand K6-TVA, WAP-TVA, MMTV-TVA and K19-TVA mice from Dr. Yi Li*
b. *Infect mammary glands of TVA mice with HIV(ALSV)-IGF1R virus (mice=60; 15 each line)*
c. *Harvest and analyze mammary glands and tumors by IHC for IGF1R and lineage markers and by FACS for luminal, myoepithelial, and progenitor cell markers*
d. *Analyze a subset of tumors for 3D organoid formation in Matrigel to determine functional capacities of lineage-derived tumors*

These TVA mice have been requested from our collaborator in Baylor. We previously requested the MMTV-TVA mice and have begun to breed these for experimentation.

Aim 3: Examine whether IGF1R expression in human breast cancers correlates with tumor initiating and cell fate markers (months 30-36)

3a: Examine the correlation of identified cell fate signaling pathways induced by IGF1R with human breast cancers expressing IGF1R

Nothing to Report

- **What opportunities for training and professional development has the project provided?**

Training Activities:

Primary Mentor for Undergraduate Student (4 semesters August 2013-May 2015): I served as the primary mentor for a University of Pittsburgh, Chemistry Pre-Medicine, student during her Junior and Senior years. I trained and mentored her alongside me in completing the more basic tasks for this grant. She is now graduating

Teacher and Mentor for University of Pittsburgh Cancer Institute High School Summer Academy (2014): I used the knowledge learned through this work, specifically the mouse work, to teach a class of summer high school students various mouse techniques with explanations on why different experimental systems is beneficial for answering different questions. Over the summer there were many STEM teaching and mentoring interactions with these students.

Professional Development:

Workshops:
Women in Medicine and Science 2 day Forum: Self-promotion (2014)

Courses/Certifications:

I am currently performing a TAR (Teaching as Research) project in the STEM field to obtain Pitt-CIRTL certification (Center for the Integration of Research, Teaching, and Learning).

Conferences:

PA BIO: Life Sciences Future

Career Development Seminars

The Academic Office for Career Development and the Women In Bio programs both have monthly seminars. For example, I attended:

How to plan for a K award
How to Negotiate
Practice your leadership: Discover your leadership style

Committee participation:

WCRC Retreat Planning Committee
Women In Bio

- **How were the results disseminated to communities of interest?**

Outreach activities:

I have participated in numerous activities and events in order to promote science to children of various ages. During these interactions, I always explain (in basic terms) what I do for a career and why it is important.

a. Summer Library Program: Science Show & Demonstration
    i. Carrolltown Public Library, PA (July 2015)
    ii. I was personally asked to perform a science showcase to 6-9 year old summary library participants during a one day visit to promote the exploration of science.
b. Science Fair Judging:
    i. Pittsburgh Gifted Center (Spring 2014)
    ii. Pittsburgh Regional Science & Engineering Fair (Spring 2014 & Spring 2015)
        1. In addition to normal judging, I was 1 of 2 people responsible for choosing 2 young female students for $50 sponsorship awards through my involvement with Young Women In Bio Pittsburgh.

Active Non-Academic Memberships:

Women In Bio, Pittsburgh Chapter: Co-Vice Chair of Communications:

I was recently asked to join the leadership team for the Women In Bio Pittsburgh chapter as the vice-chair of communications. Prior to becoming part of the leadership team, I attended numerous career development events and even helped organize a few. This very interactive organization is a great place to meet potential contacts and collaborators both within academia and industry. I have met and become acquaintances, even friends, with many WIB members ranging from students and postdocs to senior

faculty and owners of bio-tech companies. Events have included topics such as: "How to Negotiate" and "Practicing Leadership: Discover your leadership style."

- **What do you plan to do during the next reporting period to accomplish the goals?**

For Aim1, we have made new cell lines to help overcome lack of growth in the mammosphere experiments and the transfection of knockdown vectors. We will finish testing IGF1R-induced sphere formation over many passages. We will complete the siNFkB and siSnail knockdowns to finish testing the necessity of these two proteins in IGF1R induced sphere self-renewal and expansion of the CD44+ CD24- population. We will also finish testing the tumor initiating phenotypes of IGF1R in the HMEC and mouse MEC models.

For Aim2, we now have an optimized system for larger scale mouse-related experiments. We will isolate mouse MECs, infect with IGF1R virus, and test these cells in vitro for lineage potential. We will complete our testing of IGF1R-promoted mammary gland reconstitution and analyze what lineages are driven during this outgrowth. Once complete, we will then perform the more technically involved task (already optimized) of sorting the normal lineages, infecting with IGF1R, and performing reconstitution assays to test the IGF1R-induced cell fate in each mammary lineage type. While these set of mouse experiments are being performed, we will also begin experimentation of IGF1R-promoted lineage traits utilizing the TVA promoter set of mice. The end result of these mouse experiments may run into year 3 as they have long growth durations.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

A major impact upon breast cancer treatment has come from understanding the biology of the disease. Breast tumors show tremendous heterogeneity in both cell type and molecular markers. Varying molecular alterations and divergent cells of origin contribute to this heterogeneity. Our inability to prevent recurrences suggests there is a higher complexity in tumor initiation and progression than we currently understand. This tumor evolution is dependent upon a cell's capacity to evade treatment by adjusting its molecular fate. To make a major impact upon breast cancer outcome, we need to efficiently and completely kill all tumor cells, leaving little to no chance of relapse. To do this, we must first understand the drivers of tumor cell fate and the role of tumor cell differentiation in this process. The cell of origin, the cell lineage which acquires the first oncogenic hit, may greatly influence breast tumor cell fate. The capability of a tumor cell to evolve/adjust its molecular cell fate may depend on the capacity of the originating breast cell type. The cell of origin may also determine the extent to which a genomic modification can re-direct the differentiation and cell fate of a tumor cell. A tumor's exiting molecular subtype(s) and potential to change molecular fate may greatly affect how a breast tumor cell responds to treatment. We need to understand what role the breast tumor cell of origin plays in driving tumorigenesis, in generating breast tumor subtype, and in defining treatment outcome. Through this research, we strive to improve the understanding of how tumors originate, the cell lineages involved, and the genes and pathways responsible for driving the cell fate of the tumors so that novel therapies can be applied. We expect the findings from this research will improve the base knowledge for clinical application. As therapies may induce alterations in tumor cell fate, understanding the potential molecular and fate changes a tumor cell can undergo, depending on its origin,

may provide the groundwork for the development of treatments, or a series of treatments, that target the molecular alterations involved in the individual steps of resistant progression.

- **What was the impact on other disciplines?**

  Nothing to Report

- **What was the impact on technology transfer?**

  Nothing to Report

- **What was the impact on society beyond science and technology?**

  As part of the Women's Cancer Research Center, we do a fair amount of community outreach for education and fundraising. As part of this, we often bring outside members of the community into the lab, explain to them our individual projects in simplistic terms, help them to understand what research we do, and how the research impacts women's health. This improves public knowledge and attitudes.

  Additionally, through my teaching and community involvement, with both children and adults, I often discuss what research I perform. I attempt to explain in simplistic terms to increase the understanding of we scientists are trying to accomplish. These frequent interactions help to improve public knowledge and attitude about the sciences as well as the importance of the research.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  We have intent to slightly adapt Aim1, subaim1a, Task2. This task is to test the in-vivo tumor initiating ability in the absence of Snail and NF-kB activity. MCF10A cells are not easily transformed. MCF10A-CD8IGF1R cells do form tumors in immunocompromised mice, which is why this experiment was originally proposed, but the tumors are very small and soft. They do not grow robustly. We can run a pilot experiment to test the reversal of this growth upon knockdown of Snail and NF-kB, but we do not believe the tumors are robust enough to test a substantial difference in growth. We are in the process of finding another cell line model to test in vivo requirement for Snail and NF-kB. This will be tested with the normal HMECs and mouse MECs as part of the project so this piece is already duplicative, but we did originally intend to perform these experiments in the MCF10As as well. We are currently looking for a breast cancer cell line model which depends on IGF1R for tumorigenesis to replace the MCF10A model and will test requirement of Snail and NFkB for this pathway.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  The data we have thus far strongly supports the role of IGF1R in promoting tumor initiating properties and the requirement for Snail and NF-kB; however, we have not yet accomplished all of the tasks in Aim1. We have struggled enumerating the mammospheres over several passages as well as consistent

knockdown of Snail and NF-kB in our analyses. We believe the cells have low levels of mycoplasma that may be enhanced in these experimental procedures. To circumvent this, we have remade the IGF1R overexpression in new MCF10A parental cells using the pHIV-ZSG virus developed as a part of Aim2 (see below). These cells have been preliminarily tested and appear to be functioning similarly with IGF1R inducing expansion the population of cells with stem-like characteristics in a Snail and NF-kB manner (Figure 4). We will use these cells to complete the mammosphere enumeration and knockdowns. Additionally, we are purchasing viral siRNA vectors (already allotted for in budget) which will circumvent the low transfection rates of MCF10A cells. In the meantime, to keep the project progressing on time as we work out the kinks in Aim1, we have started working on Aim2 ahead of the projected schedule.

- **Changes that had a significant impact on expenditures**

Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report for all of the below:

  - **Significant changes in use or care of human subjects**
  - **Significant changes in use or care of vertebrate animals.**
  - **Significant changes in use of biohazards and/or select agents**

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**
  - **Journal publications.**

Farabaugh, S.M., Boone, D.N., Lee, A.V. (2015) Role of IGF1R in breast cancer subtypes, stemness, and lineage differentiation. Frontiers in Endocrinology: Accepted. Federal support acknowledged.

  - **Books or other non-periodical, one-time publications.**

Nothing to Report

  - **Other publications, conference papers, and presentations.**

Presentations:

UPCI Annual Retreat; Greensburg, PA: *IGF1R-Induced Changes in Mammary Cell Fate and EMT in Breast Tumorigenesis:* Poster (July 2014)

WCRC Annual Retreat; Nemacolin Woodlands, PA: *IGF1R-Induced Changes in Mammary Cell Fate and EMT in Breast Tumorigenesis:* Poster (Nov 2014)

- **Website(s) or other Internet site(s)**

  Nothing to Report

- **Technologies or techniques**

  Nothing to Report

- **Inventions, patent applications, and/or licenses**

  Nothing to Report

- **Other Products**

  Plasmids:

  Cloned pHIV-CD8IGF1R-ZSG vector

  Cell lines made:

  MCF10A overexpressing CD8IGF1R (ZSG)
  HMECS overexpressing CD8IGF1R (ZSG)

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Lauren Walheim
Project Role:	Chemistry pre-medicine undergraduate student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	13
Contribution to Project:	Ms. Walheim has performed work in genotyping, processing mouse samples, analyzing new cells lines for Aim1b, as well as repeating a few experimental procedures testing tumor initiating characteristics outlined in Aim1.
Funding Support:	No funding. Research conducted for research credit.

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

  Nothing to report

- **What other organizations were involved as partners?**

Nothing to report

8. **SPECIAL REPORTING REQUIREMENTS**

- **COLLABORATIVE AWARDS:**

Nothing to report

- **QUAD CHARTS:**

Nothing to report

9. **APPENDICES:**

Nothing to report